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Study of the potential of peptide amphiphile micelles as nanocarriers for drug delivery into the brain

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Resumo

Nos últimos anos, tem havido um enorme interesse no desenvolvimento de fármacos para o tratamento de diversas doenças. Estima-se que cerca de 1.5 mil milhões de pessoas em todo o mundo sofrem, ou virão a sofrer, de algum tipo de distúrbio do sistema nervoso central (SNC), existindo, por isso, uma grande necessidade de encontrar formas de tratar estas doenças, as quais ainda permanecem difíceis de compreender.

A libertação de fármacos para o cérebro tem sido considerada como uma possível terapia para o tratamento de tumores cerebrais e de doenças neurodegenerativas. No entanto, o acesso ao cérebro, pela via endovenosa, é bastante limitado devido à existência de uma barreira altamente dinâmica e de permeabilidade selectiva, a barreira hematoencefálica (BHE), que protege o cérebro, controlando a entrada de solutos no SNC.

Com base em Nanotecnologia, nanopartículas multi-funcionais com tamanho controlado e geometria uniforme têm sido desenvolvidas como sistemas de libertação de fármacos, com o aumento do local dos fármacos nos locais alvo, minimizando os seus efeitos colaterais.

Este projeto centra-se sobre o desenvolvimento de um sistema à base de péptido, para formar nanoestruturas do tipo micelar, com o objetivo de estudar o seu potencial para atuar como nanotransportador e conduzir fármacos para o cérebro.

O tamanho e a estabilidade das micelas são características fundamentais a ter em conta neste estudo, assim como, ensaios de libertação de fármacos e estratégias para melhorar o sistema, como por exemplo, direcionando-o para o local de destino.

Abstract

In recent years, there has been a considerable interest in developing drugs for the treatment of various diseases. It is estimated that about 1.5 billion people worldwide will suffer from some type of central nervous system (CNS) disorder at some point, so, there is a strong demand to treat these diseases, which still remain difficult to understand.

Drug delivery into the brain has been recognized as a possible therapy to treat brain tumors or neurodegenerative diseases. However, the access to the brain, intravenously, is a great challenge, due to the highly selective and dynamic permeability barrier, which protects the brain controlling the entry of solutes into the CNS, the blood-brain barrier (BBB).

Based on nanotechnology, nanoparticles with controlled size, uniform shape and carrying multi-functionalities have been developed as drug delivery systems, increasing the drugs local concentration at target sites and minimizing their side effects.

This project focus on the development of a peptide-based system, able to form micelle-like nanostructures in order to study their potential to serve as nanocarrier and deliver drugs into the brain

The size and stability of the micelles are essential features to be considered in this study, as well as drug delivery assays and strategies to improve the system, for example, directing it to the target location.

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Contents

Resumo ..... *iii*

Abstract ..... *v*

Acknowledgement ..... *vii*

Contents ..... *ix*

List of figures ..... *xii*

List of tables ..... *xiv*

List of Acronyms ..... *xv*

Chapter 1 ..... *1*

Introduction **1**

1.1. Blood Brain Barrier (BBB) 3

1.1.1. Physiology and biology 3

1.1.2. Transport mechanisms across the BBB 4

1.1.3. Changes in BBB in pathological or disease conditions 6

1.2. Nanocarriers as drug delivery systems 7

1.2.1. Inorganic nanocarriers 8

1.2.1.1. Gold nanoparticles 8

1.2.1.2. Superparamagnetic iron oxide nanoparticles (SPIONs) 8

1.2.1.3. Silica nanoparticles 9

1.2.2. Organic nanocarries 9
1.2.2.1. Polymer-based systems 9
1.2.2.2. Lipid-Based Systems 10
1.2.3. Summary of nanocarriers for drug delivery 12
1.2.4. Strategies to improve the functionality of the nanocarrier 15
1.2.4.1. Half-life circulation 16
1.2.4.2. Molecular Targeting 16
1.2.4.3. Stimuli-responsiveness 18
1.2.5. Nanocarriers mechanisms to release drugs upon reaching CNS 18
1.2.6. "Self-assembly" in nanocarriers design 20
1.2.6.1. Peptide Amphiphile as nanocarrier formed by self-assembly 21
1.3. Aim of the thesis 23
Chapter 2 ..... ***24***
Materials and Methods 24
2.1. Peptide Synthesis 24
2.2. Peptide characterization and purification 28
2.2.1. High Performance Liquid Chromatography (HPLC) 28
2.2.2. Electrospray Ionization Mass Spectrometry (ESI-MS) 31
2.3. Critical micelle concentration 32
2.3.1. Fluorescence spectroscopy 33
2.3.2. Zetasizer Nano ZS 35
2.3.2.1. Size Measurement by Dynamic Light Scattering 36
2.3.2.2. Zeta Potential by Electrophoretic Light Scattering 38
Chapter 3 ..... ***41***
Results and discussion 41
3.1. Peptide Synthesis and characterization 41
3.2. Fluorescence Spectroscopy 44

3.3. Zetasizer nano ZS.....46
3.3.1. Size measurements 46
3.3.2. Zeta potential measurements 46
Chapter 4.....***47***
Conclusions and future works..... **47**
References.....***49***

List of figures

Figure 1.1- Representation of BBB and other components of neurovascular unit including circulating cells. Adapted from [11] 4

Figure 1.2- Transport mechanisms across the BBB. Adapted from [14] 6

Figure 1.3- Nanocarriers with surface-modification. Representation of drugs (can be incorporated within the core (payload) or conjugated to the surface), targeting molecules(antibodies, cationic molecules, peptide, aptamer), PEG to enhance circulation time and fluorescent probes as a tracer. Adapted from [1] 15

Figure 1.4 – Endocytic mechanisms for targeted drug delivery to the brain, using conjugated nanocarriers. Adapted from [36] 19

Figure 1.5 - Chemical structure of a representative PA molecule with their four key domains. Adapted from [42] 21

Figure 1.6 – Illustration of nanofibre resulted from self-assembly of IKVAV-containing PA molecule. Adapted from [42] 22

Figure 2.1 - Generalized chemical formation of an amide bond. (R) side chain of first amino acid; ($\boldsymbol{R1}$) side chain of the second amino acid; (COOH) carboxylic group; (NH_2) amino group 24

Figure 2.2 - Structure of Lysine with protecting groups. Fmoc-Lys(Boc)-OH 25

Figure 2.3- Scheme of main Steps of SPPS 26

Figure 2.4 - Structure of $C_{16}K_3$.peptide 27

Figure 2.5 - Scheme of HPLC system. Adapted from [45] 29

Figure 2.6 - Representation of a Reverse-Phase Chromatography column showing three analyte bands with different speeds of elution. Adapted from [45] 30

Figure 2.7 - a) Components of ESI-MS. All the components are kept at very high vacuum since the ions in gas-phase are very reactive and have short life [47]; b) Mass spectrum of a peptide in positive and negative ionization mode [46] 31

Figure 2.8 – Behaviour of amphiphilic molecule in aqueous environment. A) Structure of amphiphilic molecule; B) Amphiphilic molecule on air-water interface; C) Micelle with hydrophobic tail inside. Adapted from 32

Figure 2.9 - Jablonski diagram representing the absorption of light (purple); vibrational relaxation, where no photon is emitted and the energy is dispersed to the solvent as heat or vibrations (green) and fluorescence (yellow) 33

Figure 2.10 - Fluorescence Spectrometer LS55- QMUL 33

Figure 2.11 – Scheme of instrument status representing the individual components and current setup of the instrument 34

Figure 2.12 - Sample concentrations (mM) analysed by fluorescence spectroscopy 35

Figure 2.13 - Malvern Instruments Zetasizer Nano ZS- QMUL..... 35

Figure 2.14 - Correlation function related with size of particles. Adapted from [56] 37

Figure 2.15 - Typical size distribution plot, showing size classes in X axis and the intensity of scattered light in Y axis. Adapted from [56]..... 37

Figure 2.16 - Scheme of the charged surface of a particle in aqueous medium. Adapted from [59] 38

Figure 2.17 - Measurement of velocity of particles moving through a fluid in an eletrophoresis experiment by laser doppler velocimetry 39

Figure 2.18 – Disposable folded capillary cell.(DTS1060) Adapted from [56]..... 40

Figure 3.1 – Chromatogram (analytical HPLC) of the crude peptide 42

Figure 3.2 – Chromatogram (analytical HPLC) of the pure peptide 42

Figure 3.3 – Mass spectrum of $C_{16}K_3$ PA, after peptide purification 43

Figure 3.4 - Fluorescence measurements of samples with concentrations between 0,002 mM and 10 mM showing different fluorescence intensity scale, a) in a range 0-140 AU; b) 0-20 AU 45

Figure 3.5 - Offset of I_max and WL at I_max vs Log(Concentration at mM), using OriginPro 9 software 45

Figure 3.6 – Intensity stands for particle size distribution and the details of the peaks 46

Figure 3.7 – Graph of Zeta potential distribution and results section giving the peaks information and zeta potential value ..... **Error! Bookmark not defined.**

List of tables

Table I - Different classes of nanocarriers, their main applications, advantages and limitations 12

Table II - Zeta potential values and correspondent stability behavior of the colloid. Adapted from [61] 39

Table III – Expected and observed mass to charge ratio of the main fragment ions of $C_{16}K_3$ peptide 43

List of Acronyms

ABC	ATP Binding Cassette
AJs	Adheren Junctions
AMT	Adsorptive-Mediated Transcytosis
ATP	Adenosine Triphosphate
AU	Arbitrary Unit
BBB	Blood Brain Barrier
BECs	Brain Endothelial Cells
Boc	*tert*-Butyloxycarbonyl
CAC	Critical Aggregation Concentration
CNS	Central Nervous System
CMC	Critical Micelle Concentration
CT	Computed Tomography
DCM	Dichloromethane
DLS	Dinamic Light Scattering
DMF	Dimethylformamide

EAK16-II	16-residue peptide
ELS	Electrophoretic Light Scattering
EPR	Enhanced Permeability and Retention
ESI-MS	Electrospray Ionization Mass Spectrometry
Fmoc	9-Fluorenylmethoxy-carbonyl
GLUT1	Glucose transporter 1
TFA	High Performance Liquid Chromatography
INEB	Instuto Nacional de Engenharia Biomédica
LAT1	Large Neutral Amino acid Transporter 1
LDLR	Low Density Lipoprotein Receptor
mAb	Monoclonal Antibody
MRPs	Multidrug Resistance Proteins
NPs	Nanoparticles
PA	Peptide Amphiphile
PEG	Poly(ethylene glycol)
PGA	Polyglicolides
P-gp	p-glycoprotein
PLA	Polyactides
PLGA	Poly(lactide-co-glycolides)
QMUL	Queen Mary University of London
RMT	Receptor-Mediated Transcytosis
SLN	Solid-Lipid Nanoparticles

SPIONs	Superparamagnetic Iron Oxide Nanoparticles
SPPS	Solid-Phase Peptide Synthesis
TFA	Trifluoro Acetic Acid
TfR	Transferring Receptor
TIS	Triisopropylsilane
TJs	Tight junctions

Symbols

β	Beta

Chapter 1

Introduction

Due to significant advances in healthcare technologies, people are living longer contributing for the increase of an aged population. Associated to this aged population, is a high incidence of neurological disorders, which have an enormous social impact and are an important cause of morbidity, contributing to 12% of global deaths. [1]

Neurological disorders are any disorder of the body's nervous system which can include neuropsychiatric disorders and injuries that have neurological sequels. [1] Neurological disorders within the neuropsychiatric group, like epilepsy, Parkinson's disease, multiple sclerosis, Alzheimer and other dementias, are the main cause of mental, physical and social disability, affecting a great number of persons.

Since there are no effective therapies for many of them yet, [1] probably due to the inadequate understanding of the nervous system, treating these diseases is a major challenge and drug delivery into the brain has been recognized as a possible therapy.

Delivering drugs at controlled rate and targeted transport are emerging to solve the limitations of conventional drugs and to increase the therapeutic efficiency. Drug delivery systems have been designed to protect drugs from degradation. They must be biocompatible, so that body's defense

system does not recognize them as foreign particles and localized at target diseased areas, minimizing their side effects. [2]

Significant progress has been observed in this area, thanks to advances in nanotechnology, which uses engineered materials or devices, typically ranging from 1 to 100 nanometers that can interact with a biological system with a high degree of specificity. [3]

Drug delivery systems have been classified according to the routes of delivery, type of delivery vehicles and the employed strategies to reach the target. [4]

Drugs can be administered in different ways depending on the type of disease, the desired effect and the available product. [5] Routes of administration can be classified whether the effect is local and the drug is applied directly to the organ affected (topical route), which is not an option in cases of CNS disorders, or systematic, targeted to the diseased organ (enteral or parenteral route).

The administration through the gastrointestinal tract is enteral, including oral, rectal or sublingual. The oral route is, generally, the most used and highly preferred due to the ease of administration and the lowest cost. However, some drugs can cause problems, like irritation of the gastrointestinal tract, can be degraded by digestive enzymes before they reach the target site or cannot effectively reach the blood stream due to their high molecular size or charge, like proteins and peptides.

The term parenteral means the introduction of drugs into the patient through intramuscular, intravenous, intra-arterial and subcutaneous injection and gets advantage of rapid onset of action and avoid the oral administration problems, mentioned above. [5, 6]

Drug delivery strategies established to act in the central nervous system (CNS) to treat neurological disorders or brain tumours have an additional barrier unlike in the other therapeutic areas, the blood brain barrier (BBB). Thus, these drugs to be administered intravenously must have the ability to cross this barrier. However, the strategies already developed are proved invasive and non-specific to the target, including osmotic disruption to increase the BBB permeability, direct introduction of the agent in the CNS or intracerebral implantation. Besides the associated high risk of complications, these procedures are rather expensive. [7]

To solve these limitations, new approaches are emerging, using nanoparticles with controlled size, uniform shape and carrying multi-functionalities in order to cross the BBB noninvasively facilitating their delivery in to the brain.

1.1. Blood Brain Barrier (BBB)

CNS has its own security system, highly selective and dynamic permeability barrier that allows the entry of essential nutrients, while blocking invading organisms and unwanted substances, the blood-brain barrier.

Although the BBB has an important function to protect the brain from toxic compounds, it is also the main challenge for drug delivery into the brain, since it represents the major barrier for drug transport into the brain via the blood circulation.

This section presents a better understanding associated to the physiology and biology of the major barrier of our body and its changes under pathological conditions.

1.1.1. Physiology and biology

Brain endothelial cells (BECs), which form the walls of capillaries, are different from endothelial cells in the other organs. They exhibit lower permeability and pinocytic activity, no fenestrations and possess a superior number of mitochondria, resulting in a more intense metabolic activity. [8]

BECs are characterized by the presence of interconnections between adjacent cells, like gap junctions that mediate intercellular communication [9], adherens junctions (AJs) and tight junctions (TJs), which are responsible for the high transendothelial electrical resistance that reduce the aqueous based paracellular diffusion [10] and prevent transcapillary passage of ions, hydrophilic compounds and macromolecules, such as proteins.

As a result of these anatomical features, these cells form a cellular barrier that constrains the passage of molecules and cells between the blood and brain.

The BBB is a natural protection of the brain against adverse agents, allowing the neurons to communicate with each other through chemical and electrical signals, and it keeps the interstitial fluid composition stable so that the neurons can maintain a controlled electric potential support. [11] The functional unit of BBB, shown in figure 1.1, consists not only by capillary endothelial cells and neurons but also by several other components such as pericytes, basal lamina components, perivascular

microglial cells, astrocytes. Pericytes and perivascular astrocytes in contact with the endothelium [12] are essential to maintain the equilibrium conditions in the interstitial space [11] and pericyte dysfunction can cause the BBB to snap leading to several brain disorders.

BBB also controls the recruitment of leukocytes and regulates the innate immune response acting as the boundary between central and peripheral immune systems. [6]

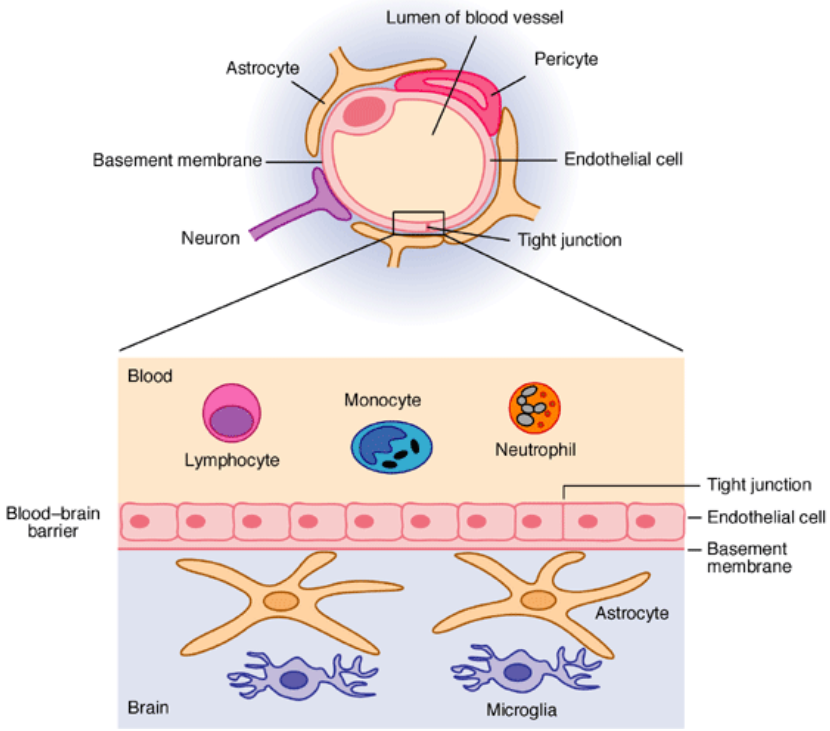


Figure 1.1- Representation of BBB and other components of neurovascular unit including circulating cells. Adapted from [11]

1.1.2. Transport mechanisms across the BBB

The unique biological characteristics of the BBB are responsible for its multiple dynamic functions acting as a transport, physical, enzymatic and immunological barrier [13] and capable of responding to a wide range of regulatory signals from both sides contributing to CNS homeostasis. [6]

There are several transport pathways by which solutes can move in and out of the brain, including passive and facilitated diffusion, which proceed from low to high concentrations, and active transport,

that requires adenosine triphosphate (ATP) as a chemical energy source and conducts movement against the concentration gradient. [14]

Diffusion of substances into the brain may be performed by passing through the intercellular space between the cells (paracellular diffusion) or through a cell (transcellular diffusion). Paracellular diffusion (figure 1.2 (***a***)) does not occur in great extent at the BBB, such as small water-soluble molecules that passively diffuse through the TJs, unlike the small lipid soluble molecules that penetrate transcellulary (figure 1.2 (***b***)) by dissolving in their lipid plasma membrane.

Besides the concentration differences between both sides, lipophilicity and molecular weight are other parameters that can affect the diffusion. Highly lipophilic and small substances (less than 500 Da) such as small inorganic molecules (O_2, CO_2, H_20), are highly permeable.

Nevertheless, poorly permeable substances require a mediated transport that facilitates movement through the BBB by carrier-mediated transport, or endocytosis/transcytosis.

In the case of carrier-mediated transport, membrane transporters, that are mostly proteins, aid the transport into the brain (by facilitated diffusion or active transport) and block the entry of several molecules.

Essential solutes such as glucose or amino acids bind to a protein transporter (glucose transporter GLUT1 or amino acid transporter LAT1) which changes its conformation allowing the traffic of these solutes to the other side (facilitated transport) (figure 1.2 (***c***)). However, there are some cases in which substances need to be moved against concentration gradient using energy provided from ATP (active transport).

ATP family of efflux transporters (ATP binding cassette- ABC) including p-glycoprotein (P-gp) and multidrug resistance proteins (MRPs) are responsible to prevent the permeability of several pharmacological agents.

In addition of these transport systems, certain endogenous proteins and peptides pass through the BBB by vesicular transport and transcytosis that can be receptor- or adsorptive- mediated.

Receptor-mediated transcytosis (RMT) such as transferring receptor (TfR), insulin receptor or lipoprotein receptor allows selective uptake of macromolecules. These processes are activated when a

ligand, for example insulin molecules, bind to cell-surface receptors invaginated into the cytoplasm forming a coated vesicle. (figure 1.2 (***d***))

Adsorptive-mediated transcytosis (AMT), or pinocytosis route, facilitates the transport of large peptides such as histone, albumin and other plasma proteins by electrostatic interactions, binding the positively charged moiety of a peptide to the negatively charged cell membrane surface. (figure 1.2 (***e***))

The transport of immune cells, such as monocytes or macrophages, across the BBB is designated cell-mediated transcytosis and can be used for any type of materials, unlike the other types of transport mechanisms. [6, 12, 13]

Many of the mentioned pathways across the BBB can influence drug delivery having reduced access to CNS when agents are less lipophilic due to the activity of ABC efflux transporters. [6]

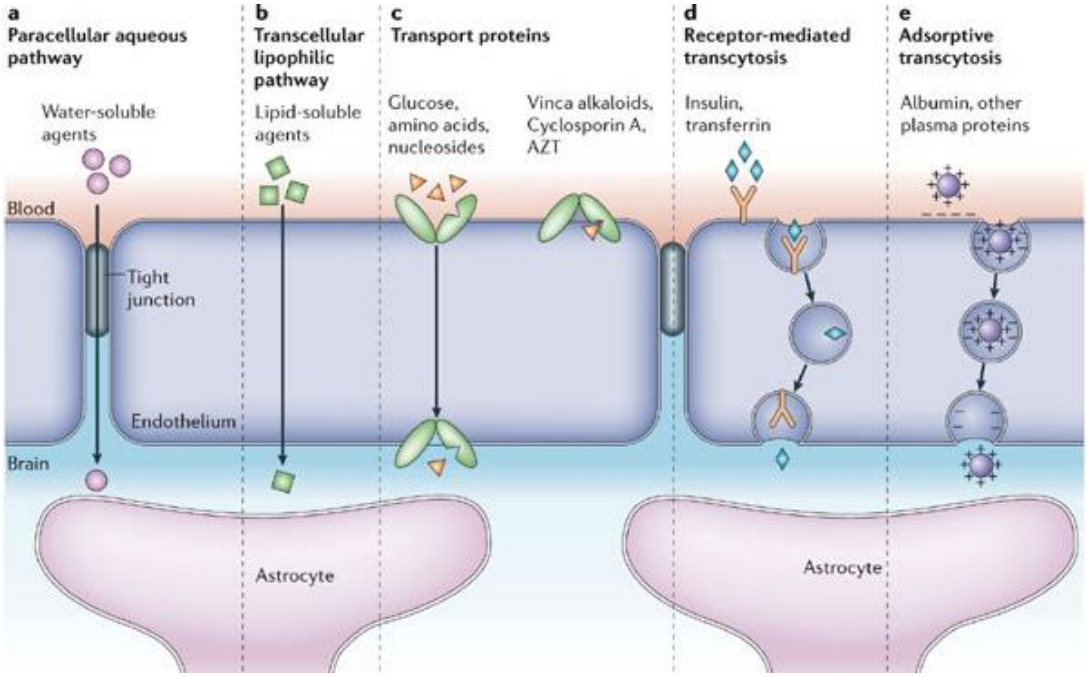


Figure 1.2- Transport mechanisms across the BBB. Adapted from [14]

1.1.3. Changes in BBB in pathological or disease conditions

The properties of the barrier can be disturbed in pathological conditions affecting its tightness and functions including transport mechanisms. [6, 13] Most CNS pathologies, including stroke, multiple

sclerosis, epilepsy, start with a sequence of events which leads to an increased BBB permeability and can contribute to disease progression. [6] These alterations of BBB permeability are possibly due to disruption of TJs, since they form the physical barrier which may be caused by an increased transmigration of leukocytes into the brain like it happens in inflammatory diseases.

The integrity of BBB remains in normal aging, but the changes in the brain vasculature may make the BBB more vulnerable to pathological injuries. [15]

In age-related neurodegenerative disorders, such as Alzheimer's and Parkinson's disease, the relationship between BBB dysfunction and pathology is not clear, although it has been suggested that it can contribute to disease progression. [16]

Thus, this barrier can be considered a target of therapeutics to treat these diseases. However, the changes are not the same for all diseases and also depend on the degree of development of each disease being difficult to predict the effects of any particular drug. [1]

Due to the complexity of the BBB on pathological conditions, this topic will not be described in this thesis, but further details can be found elsewhere. [1, 6, 15, 16]

1.2. Nanocarriers as drug delivery systems

As mentioned previously, delivery of drugs into the brain is a great challenge due to difficult passage across the BBB and several approaches have been studied to bypass this barrier noninvasively.

This section focuses on delivery vehicles, namely nanocarriers, being one of the approaches to overcome this challenge.

Nanocarriers are nanomaterials that can be used to transport drug molecules. Drugs can be bound in the form of a solid solution, dispersion, be adsorbed to the surface or chemically attached on nanoparticles. [17]

Because of their small size, they can penetrate into dense tissues, such as tumours, and can be functionalized in order to deliver drugs at target sites and at controlled rate.

To this date, several nanoparticles have been developed to act as drug carriers, including inorganic or organic materials with advantages and some limitations, depending on the purpose.

For nanocarriers to serve as good candidates for delivering drugs, many factors need to be considered such as its size (less than 100 nm); toxicity, biocompatibility and biodegradability; stability in blood; prolonged circulation time; controlled and target release, as mentioned previously.

1.2.1. Inorganic nanocarriers

1.2.1.1. Gold nanoparticles

Gold Nanoparticles (Au NPs) have been widely used for several applications due to their various size and shape. Among all the shapes, such as spherical, triangle, irregular shape, nanotriangles, nanoprisms and nanorods, the later having increased importance due to their specific optical and chemical characteristics, making them suitable for biosensing, gene delivery and photo thermal therapy. Gold NPs can also be used as molecular probes for imaging due to their high adsorption coefficient and the electron density of the gold which improves computed tomography (CT) contrast. They can be easily synthesised, present low cytotoxicity, are small sized and have uniform dispersion helping them to reach the target site. Their large surface area provides drug attachment and they were shown to be biocompatible. These are some more features of gold NPs that make them advantageous over other nanocarriers. [18] However, since it is a solid particle, encapsulation of the drug is not possible, leading to more complex techniques to attach the drug to the nanocarrier, implying more time-consuming. The major limitation of these systems, with the intention to act in the brain, is the lack of biodegradability, meaning accumulation into the brain, requiring surgical removal.

1.2.1.2. Superparamagnetic iron oxide nanoparticles (SPIONs)

These nanoparticles are composed by superparamagnetic compounds which are attracted to a magnetic field when it is applied, but when removed they do not retain any residual magnetic

interactions, being unlikely to agglomerate and posing no danger of thrombosis or blockage of blood capillaries.

Due to their paramagnetic features, they can be analysed by magnetic resonance imaging (MRI) and are advantageous for use as drug delivery vehicles because they can be guided to their target site and release the drug under the influence of an applied magnetic field.

However, there are some limitations, such as their unspecific uptake by target cells and uncoated SPIONs have demonstrated low brain-targeting efficacy.

The biocompatibility of these nanoparticles and their possible adverse interactions with the body are still under investigation. [19, 20]

1.2.1.3. Silica nanoparticles

Silica nanoparticles are nanoshells, spherical nanoparticles consisting of a dielectric core surrounded by a metallic shell. Silica is typically used as core due to its high stability. [19] The biocompatibility and the excellent protection for their internal payload make them excellent carrier for controlled drug delivery applications. [21] Though, Chang et al (2007) found that, at high dosages, silica nanoparticles could be toxic shown by a reduction in cell viability.

1.2.2. Organic nanocarries

1.2.2.1. Polymer-based systems

Polymeric nanoparticles (polymeric NPs) – These nanoparticles are composed of a core polymer matrix in which drugs can be embedded and are made from synthetic polymers such as polyactides (PLA), polyglicolides (PGA) or poly(lactide-co-glycolides) (PLGA) among others or natural polymers, such as chitosan. [1]

Some studies have proven that polymeric NPs enhance drug delivery to the brain with control release mechanisms, namely NPs made of PLGA embedding antituberculosis drugs for brain delivery, demonstrating high drug levels in the brain for more days than with free drugs. [1]

However, to cross the barrier to reach the brain, these nanoparticles have to undergo several modifications which add complexity and time to the process. Also, their large scale production is problematic.

Dendrimers – Dendrimers are highly branched polymers with a well-defined chemical structure, consisting of a core or focal moiety and multiple arms which emerge from the core, with functional terminal groups. These play a key role in the drug entrapping ability due to their specific molecular structures, tightly packed in the periphery and loosely packed in the core. [19, 22]

Thus, dendrimers can be used for drug delivery by modifying their terminal groups for drug targeting. [1] However, the rigidity and density of the branched units can affect drug release kinetics. [22]

Polymeric micelles – Polymers can also be used in drug delivery enveloping drugs through micellization. Polymeric micelles are amphiphilic copolymers formed by self-assembly in aqueous solution resulting in core-shell nanostructures with a hydrophilic shell and a hydrophobic core which can act as reservoir for entrapping water insoluble drugs.

Using micelles as vehicle in drug delivery has many advantages, including high drug loading capacity in the core and their nano-range size properties leads to a rapid cellular uptake.

However, these systems can present some specific disadvantages such as the difficulty in polymer synthesis unlike the random polymers and low drug loading capacity or difficult drug incorporation technology, mainly in large scales. [1, 2, 22, 23]

1.2.2.2. Lipid-Based Systems

Liposomes – The use of liposomes as drug carriers is another type of nanoparticle. Liposomes are vesicular structures composed of a hydrophobic lipid bilayer, made of biocompatible and biodegradable lipids, delimiting an aqueous core.

The drugs can be packed within liposomes or intercalated into lipid bilayers, enabling the liposome to carry both hydrophilic and hydrophobic drugs and to merge with cell membrane releasing the drug. [1, 17, 19, 24]

Nevertheless, the immediate uptake and clearance of the liposome by the reticuloendothelial system can be immediate can be a problem in using these *in vivo*, only being resolved with surface modifications. Hence, liposomes present low stability.

Solid-lipid nanoparticles (SLN) – These lipid-based nanoparticles contain a solid hydrophobic lipid core in which the drugs are dissolved and can be used for several therapeutics as colloidal drug carriers in the place of liposome or emulsions, since these have a better stability.

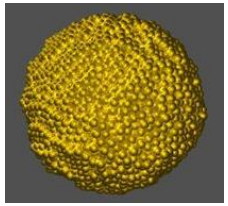
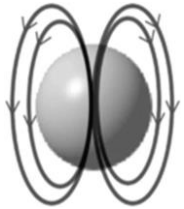
SLNs can be produced by replacing the liquid lipid oil of an emulsion process by a solid lipid and have the advantage of possessing more flexibility in controlling the release of the encapsulated drug provided by the solid matrix. [1, 17, 25]

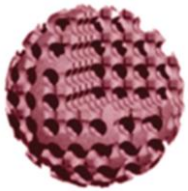
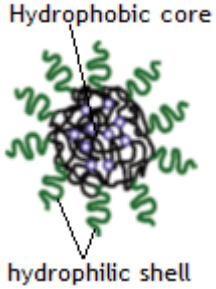
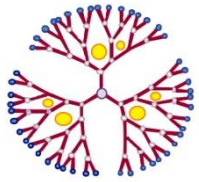
Besides these great advantages, they also can suffer from some disadvantages. The crystalline structure of the solid lipid leads to a low incorporation rate.

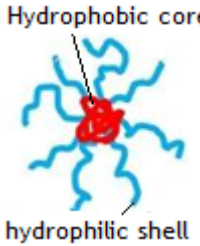
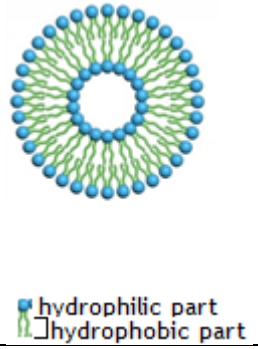
1.2.3. Summary of nanocarriers for drug delivery

Table I summarizes the main applications and main advantages and limitations of these various nanocarrier systems described in the previous section.

Table I - Different classes of nanocarriers, their main applications, advantages and limitations

Class	Main Applications	Advantages	Limitations	Schematic representation	References
Inorganic					
Gold NPs	Chemical and biological sensing Biomedical imaging Tracking and drug delivery Catalysis	Biocompatibility Non-cytotoxicity Easily synthesized Unique optical, physical and chemical properties Diverse/multiple shapes	Lack of biodegradability Solid particle, complexity in attaching the drug		[1, 18]
SPIONs	Targeted delivery of drugs/genes Thermotherapy	Easy to manufacture MRI visualization of neuroinflammation *in vivo* Monitorization of the CNS disease progression	Accumulation into the brain Unspecific uptake by target cells without coated		[1, 17, 19, 20]

Silica	Imaging Drug delivery Catalysis Blood immunoassay	Optical and chemical properties High Biocompatibility High porosity (high loading efficiency)	Toxicity Lack of biodegradability		[1, 17, 19, 21]
Organics					
Polymeric NPs	Deliver water insoluble drugs	Stability Controlled release Stimuli responsive	Lack of biocompatibility Do not undergo significant transport through the BBB without further modifications		[1, 26]
Dendrimers	Imaging Drug delivery Gene transfection Therapeutic treatment	High drug carriage Terminal groups can be modified for drug targeting	Rigidity and density of the branched units Cytotoxicity of the polymers after internalisation into cells		[17, 27]

Polymeric micelles	Drug delivery	Good grade of stability Easy conjugation for active targeting High drug loading capacity	Difficult polymer synthesis Need surface modifications to reach the target sites		[1, 2, 28]
Liposomes	Drug delivery Cosmetic delivery	Favourable safety profiles Ease of surface modifications Able to carry both hydrophilic and hydrophobic	Short half-life High production costs Unstable in biological conditions		[22, 24]
Solid Lipid NPs	Drug delivery	Stability Large surface area High drug loading Controlled release	Low incorporation rate Gelation tendency		[19, 25]

1.2.4. Strategies to improve the functionality of the nanocarrier

These nanocarriers have shown efficiency in pharmaceutical industry, however many of these can suffer from low selectivity and lack of target-ability, uncontrolled release profile and low circulation life in the blood stream. Therefore, several strategies to modify their surfaces have been developed to overcome these problems.

The most common approaches consist on the functionalization of nanocarriers to increase stability and half-life circulation, target specific tissues and making them responsive to certain physiological stimuli such as pH and temperature. [2]

Some possibilities of nanocarrier functionalization for brain drug delivery are represented in figure 1.3. [1]

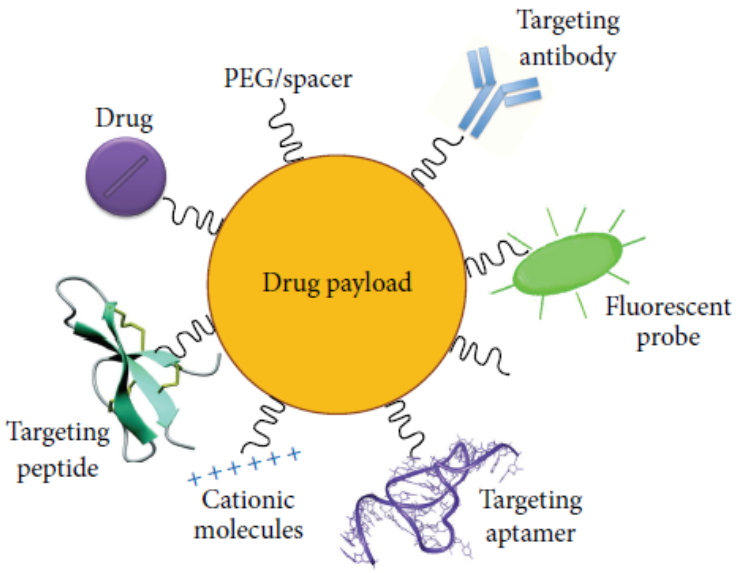


Figure 1.3- Nanocarriers with surface-modification. Representation of drugs (can be incorporated within the core (payload) or conjugated to the surface), targeting molecules(antibodies, cationic molecules, peptide, aptamer), PEG to enhance circulation time and fluorescent probes as a tracer. Adapted from [1]

1.2.4.1. Half-life circulation

One of the nanocarrier properties to take into account for drug delivery through vascular route into the brain is its stability in the blood which means no aggregation and dissociation and longevity in blood circulation since the immune system such as macrophages can recognize the nanocarriers as foreign bodies and remove them from circulation before they achieve the target site and perform their action. [2, 13]

The most widely used strategy for reduced uptake by macrophages and to solve this issue is PEGylation, the coating of the material with an highly flexible and hydrated poly(ethylene glycol) (PEG) chain. [29]

This is because Frank F. Davis in 1977 has studied the immunogenic properties of the PEG and concluded that the attachment of this hydrophilic polymer to proteins controls immunogenicity and extends circulation time in rabbit and mice. [2, 30]

Thus, surface functionalization of nanocarriers with PEG has become a stealth strategy to increase nanocarrier's circulation half-life in which PEG molecules form a hydrophilic layer on the nanocarrier's surface protecting them from interaction with blood components, designated "steric stabilization" effect. [29]

However, this effect is no longer favourable when the nanocarrier arrive the target site. The drug entrapped in a nanocarrier should be specifically delivered into the pathological sites and this coating can be an obstacle, so many approaches have been developed to detach the PEG after onset at the target sit, described below. [29]

1.2.4.2. Molecular Targeting

Another largest problem of many pharmaceutical agents is the lack of target-ability causing side effects and toxicity. Targeting strategies for drug delivery systems can be passive or active targeting.

The increased BBB permeability on pathological conditions can be a window of opportunity for drugs unable to cross this barrier to reach the target in the diseased brain. [13] This selective targeting based on permeability, like in tumor tissues leading to enhanced permeability and retention (EPR) effect, is considered "passive targeting". However, there are some limitations since the degree of BBB permeability may not be the same in all disorders and also depends on status of each disease. [31]

An “active targeting” can overcome these limitations modifying the surface of nanocarriers by covalent conjugation of targeting moieties, which only bind to specific receptors on the cell surface, leading to internalization of the nanocarrier through receptor-mediated transport, in order to ensure the drug is released in a specific site, such as CNS. [24] These receptors should be highly expressed only on specific sites so that high specificity is reached.

Antibodies and their fragments, aptamers and other receptor ligands such as peptides and vitamins are potential active agents. [22] The choice of a target is a fundamental aspect in the development of these functionalized nanocarriers and depends on several factors such as target expression which determine delivery specificity, their function and biocompatibility to control potential side effects, binding affinity as well as size and charge and ease of modification to the surface. [22, 32]

Several strategies have been developed by receptor-mediated transcytosis since many receptors are overexpressed in the brain endothelium such as TfR or low density lipoprotein receptor (LDLR).

Many research groups have been explored transferrin as a brain delivery ligand and shows anti-glioma activity when conjugated with a polyphophoester hybrid micelles (Zhang et al.). However, since this system would have to compete with natural ligand an alternative emerged such as the conjugation with a monoclonal antibody (mAb). Liposome conjugated with this system transferring/antibody, the drug targeting was achieved. [33, 34]

The mAb are the most widely used for targeting cancerous cells, approved by the US food and Drug Administration (FDA). However, due to their large size, trouble in conjugation to nanocarriers or the immunogenic response that can elicit itself, peptides and other small molecules have been an outstanding alternative to solve these limitations. [31, 35]

Another receptor widely studied for ligand mediated targeted brain delivery is the LDLR and angiopep, a peptide which display higher transcytosis capacity, so that can act as ligand for targeting to this receptor. Results demonstrated that angiopep-modified NPs crossed the *in vitro* and *in vivo* BBB models more efficiently than unmodified NPs. [33, 34]

1.2.4.3. Stimuli-responsiveness

To program its release at target sites, an additional functional group can be coupled in a multifunctional nanocarrier responsive to the physical or chemical alteration of the local site. [32]

In a pathological condition some alterations are noted such as lower pH or higher temperature, making the injured areas, like inflamed or tumor tissues, different from normal tissue. [2, 29] Thus, nanocarriers survive in normal conditions and are only subjected to degradation, with consequent drug release, in the area of interest. [29]

The stimulus to degrade the carrier can also be external, such as heat, magnetic field and ultrasound, which can be locally applied on the target tissue allowing the nanocarrier to release the drug only inside this area. [2, 29]

1.2.5. Nanocarriers mechanisms to release drugs upon reaching CNS

After reaching the target site, it is important to know how these nanocarriers release the drugs, being the mechanisms still not well understood depending on functionalization, dosages, via of administration, since the environment of CNS and BBB change in case of disease.

The main mechanisms already studied in targeted brain delivery involves active targeting, using nanocarriers conjugated to site-specific ligands, being recently reviewed by Bareford et al. the role of endocytosis, which can improve the treatment of diseases like Alzheimer.

Figure 1.4 represents the two main mechanisms of endocytosis mediated transport of nanocarrier systems: receptor-mediated and adsorptive-mediated. Nanocarriers conjugated to ligands which bind to specific receptors on BECs can be internalized via receptor-mediated (1) where they are invaginated forming vesicles which, due to acidification, dissociate the receptor from the ligand leading to the release the drug and the degradation of the nanocarrier (1b).

The adsorption of uptake promoting apolipoproteins have been proposed as other mechanism, concluded by Kreuter et al that apolipoproteins B and E can adsorb the coating nanoparticles with polysorbate 80 and, via receptor-mediated endocytosis, could be taken up by BECs, since mimic lipoprotein particles.

Adsorptive-mediated has also gained significance in drug transport, using cationized ligand conjugated nanocarriers to interact with surface of BECs which are negatively charged. Dos Santos et

al. found heparin sulphate (HS), a polyanion detected on BCEs surface, but more investigation is required to explore other polyanion sites to develop these systems.

Lu et al., has incorporated plasmid pORF-hTRAIL (pDNA) into cationic albumin-conjugated PEGylated nanoparticles (CBSA-NP) and observed that these NPs co-localized with glycoproteins in brain and accumulation in tumour cells that led to their apoptosis and, significant delayed tumour growth. [36]

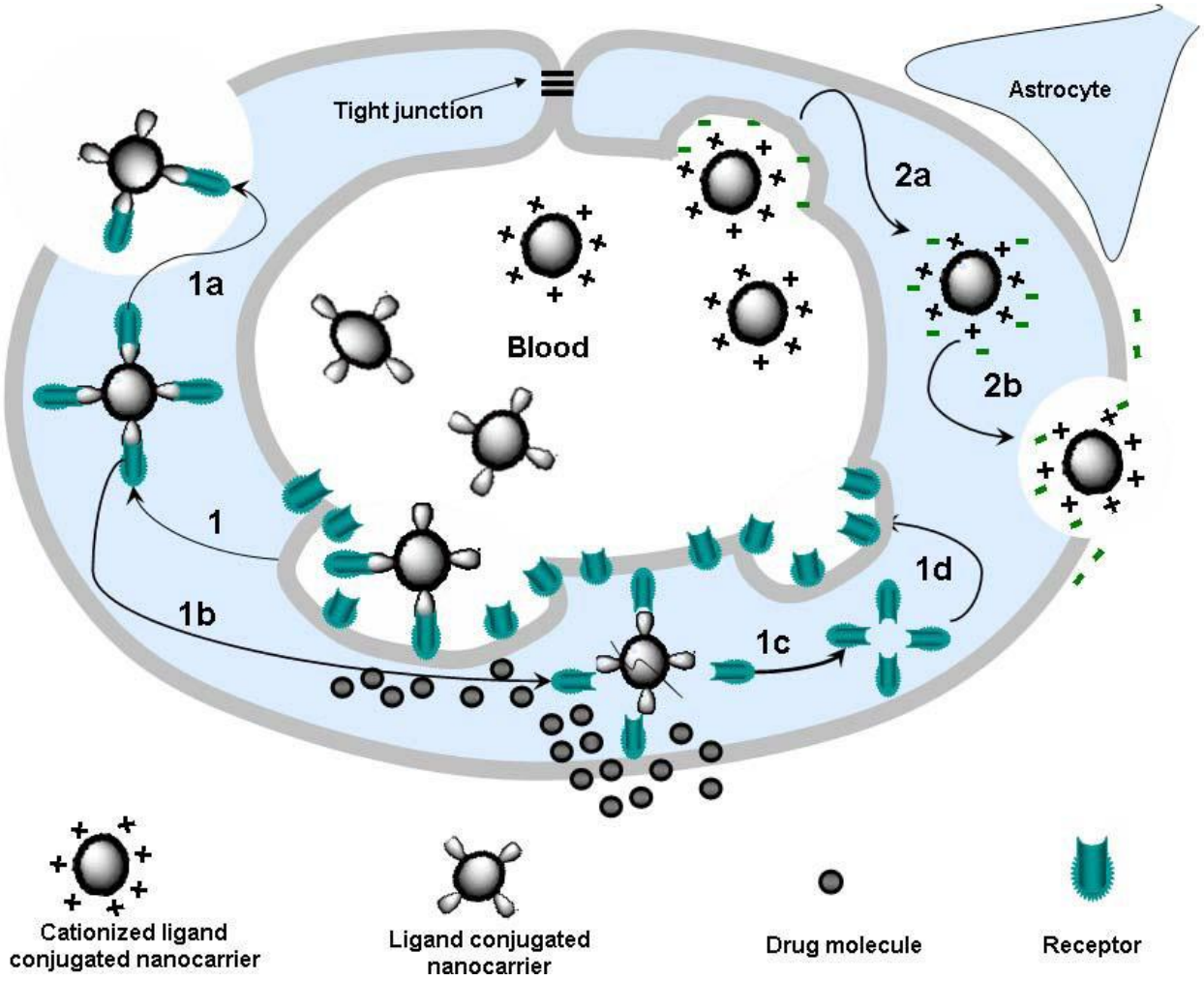


Figure 1.4 - Endocytic mechanisms for targeted drug delivery to the brain, using conjugated nanocarriers. Adapted from [36]

Some drugs can also be delivered into the brain by carrier-mediated mechanism, since the membrane transporters presented in the BBB have been shown to accept drug molecules and to carry them into the brain. However, it is also important to take into consideration the efflux transporters which can exclude the drugs out of brain. It is, therefore, essential to identify all the transporters to inhibit them improving the drug distribution in the brain. [37]

1.2.6. "Self-assembly" in nanocarriers design

Molecular self-assembly is the organization of molecular units in a spontaneous, reversible and coordinated fashion to form complex structures by non-covalent interactions (hydrogen bonding, electrostatic and hydrophobic interactions). Many of these interactions occur in biological systems, such as the tertiary and quaternary structure of proteins.

Inspired on nature, self-assembly has been increasingly studied to construct functional nanomaterials to cover a wide variety of applications, allowing a better structures and functional control at the molecular level. [38, 39]

The self-assembled systems provide great advantages being a simple and fast process. Their ability to generate well-ordered 3D structures with sub-nanometer precision, due to operating at molecular level, without a guidance of external source, also makes it an attractive "bottom-up" strategy.

Unlike the systems mentioned above, Brownian motion, the random movement of the particles in a liquid due to the collision with molecules which surround them, does the job instead of machinery to move and orient components. [40]

Due to these advantages, many advances have been done in the design and fabrication of nano-scale materials trough peptide self-assembly.[39]

The simple structure of peptides, their stability under various conditions and their variety of self-assembled shapes make them the best choice for design different materials using natural building blocks. [41]

1.2.6.1. Peptide Amphiphile as nanocarrier formed by self-assembly

Peptides are made of amino acids, considered natural building blocks, which due to their different chemical nature (polar, non-polar, positively or negatively charged, aliphatic, etc.), can form a large number of different peptides, through the combinations of different amino acids lengths and sequences. [39]

A simple category of the designed self-assembling peptides is represented by molecules that combine the structural and chemical properties of lipid or surfactant with the biofunctional peptide epitope and have the ability to self-assemble into a wide range of different nanomaterials. These peptide-based molecules are called peptide amphiphile (PA) and have gained wide attention because of their strongly amphiphilic nature, possessing a hydrophobic tail and hydrophilic head. [38, 39]

In aqueous environment, these peptides can form structures aggregates such as nanofibers, nanovesicles, nanotubes, through self-assembly when the concentrations are above their critical aggregation concentrations (CAC) which can be detected by fluorescence methods.

Figure 1.5 represents the requirements for a functional PA to assemble into a well-defined structure, i.e., with different regions, each with their role, that make it to be formed structures in an organized and orderly way. Region 1 includes the hydrophobic entity, consisting of a hydrophobic segment made from non-polar amino acids remains or alkyl chains. This region is responsible for driving self-assembly due to hydrophobic interactions between different tails, exposing the functional peptide group on the surface of the nanostructure.

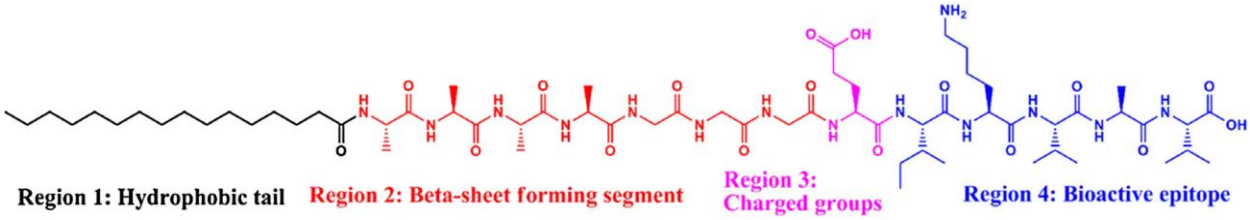


Figure 1.5 - Chemical structure of a representative PA molecule with their four key domains. Adapted from [42]

The hydrophobic alkyl tails also improve the thermal stability of the PAs, being PAs with longer tails more stable but less soluble. A study about self-assembly of PAs containing RGD cell adhesion motif, shows that PAs with only six carbon atom alkyl tails were not able to form gel, unlike PAs with C10, C16 and C22 which formed gels upon acidification. [42]

For further aggregation to occur, region 2, which have the ability to form intermolecular hydrogen bonds between the peptide strands consisting of a peptide sequence leading to ß-sheet formation, determines the interfacial curvature of the self-assembled structure. Systems without this region and so, where there are only hydrophobic interactions, lead to formation micelles with the hydrophobic tails inside the core and the hydrophilic heads exposed to water, due to the dislike of tails to water.

The influence of stability concerns the region 3, charged amino acids residues can be positively, negatively charged or a mixture of both. Therefore, the self-assembly of PAs can be altered changing the solution pH.

The last region is where bioactive signals are presented through functional peptide epitope which can interact with desired target. [38, 39, 42]

One example of aggregation of these PA molecules having these four domains is the formation of cylindrical nanofibre, as shown in figure 1.6

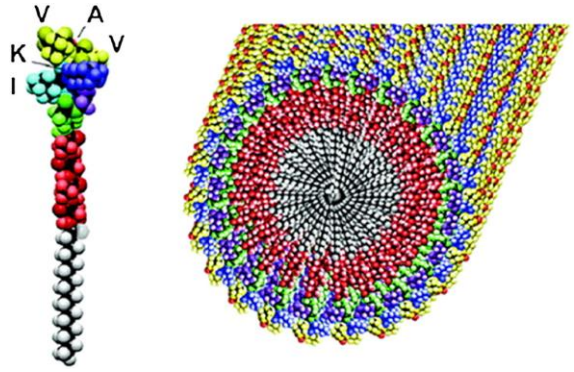


Figure 1.6 - Illustration of nanofibre resulted from self-assembly of IKVAV-containing PA molecule. Adapted from [42]

The self-assembly of PAs can be tuned and manipulated in response to a stimulus by changing the local environment, being useful in the production of biomaterials and in a wide range of applications such as drug delivery systems.

For all these reasons, these systems represent a better option when the focus is the design of a nanocarrier, having great advantages over the others nanocarrier systems, mentioned previously. The self-assembly of PAs leads to different functional nanostructures with multiple properties such as targeting ability, without complex and time-consuming techniques as in others.

These four main regions, each with their key role, incorporated into PAs makes them an interesting systems for drug delivery into the brain, without the need for further modifications on the surface.

1.3. Aim of the thesis

Therefore, this project aimed to design and develop a better nanomaterial able to cross the BBB and reach the brain in order to deliver drugs at controlled rate and target transport, minimizing their side effects.

Peptide amphiphile was synthesized to form micelles, in aqueous environment, by self-assembly. The micelle conditions, such as their size and stability, were studied to understand their potential, to act as nanocarrier, in drug delivery into the brain.

Chapter 2

Materials and Methods

2.1. Peptide Synthesis

Peptide synthesis has been an important tool in medical areas since synthetic peptides can be used as therapeutic drugs or in diagnosis.

Peptides can be synthesized by attaching the carboxylic acid group (C-terminus) of one amino acid to the amino group (N-terminus) of another amino acid forming an amide bond, as shown in figure 2.1. This process is called coupling. [43]

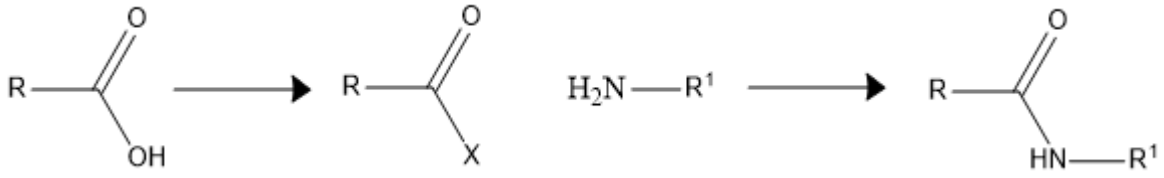


Figure 2.1 - Generalized chemical formation of an amide bond. (R) side chain of first amino acid; ($\boldsymbol{R^1}$) side chain of the second amino acid; (COOH) carboxylic group; (NH_2) amino group

This procedure is not so simple since the diversity of the various amino acids can originate numerous different combinations. To make sure that the peptides are formed with the desired chain in the right order, the amino acids side groups should be protected to prevent side reactions. The mostly used protective group is *Fmoc* (9-fluorenylmethoxy-carbonyl), which is used for the amino protection of the

N-terminal In addition, many amino acid side chains must be protected as well as in the case of Lysine with *Boc* (a tertiary Butyloxycarbonyl group), for not forming undesired branched chains.

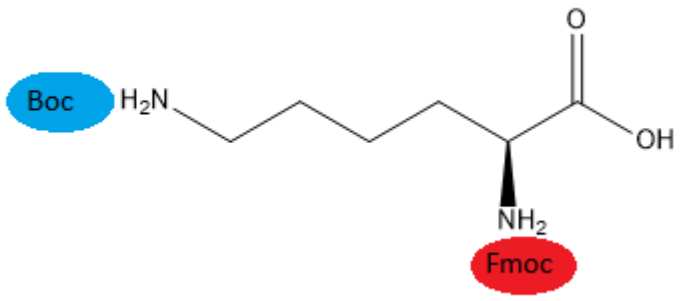


Figure 2.2 - Structure of Lysine with protecting groups. Fmoc-Lys(Boc)-OH

The classical approach to synthesize peptides is the synthesis in solution which has the advantage to detect undesirable side reactions since, at every step, the intermediate compounds can be characterized and isolated. However, the low yield of the intermediate peptides and the issue of poor solubility are limitations of this method.

In order to solve these problems, another method was developed where insoluble polymers are used as a support for the peptide synthesis, known as solid-phase peptide synthesis (SPPS).

SPPS is simple, faster and high yield approach than the classic solution phase and solves the problem of solubility since the growing peptide chain is only detached from the support when the process has been completed. In addition, it can be made by an automated peptide synthesizer leading to a good and quick performance or manually, in the case of the miligram scales. [43, 44] For that reason, SPPS it was the method of choice in this work.

The peptides undergo several steps until are purified. First, the peptide protected at its N-terminus, and its side chain when it is necessary, is attached chemically to a solid polymer, Rink Amide- MBHA resin in the case of this work, following repetitive cycles of deprotection and coupling to a next amino acid, until have a synthesized desired peptide. Then, the peptide is ready to be cleaved from the resin to be purified and characterized. [43]

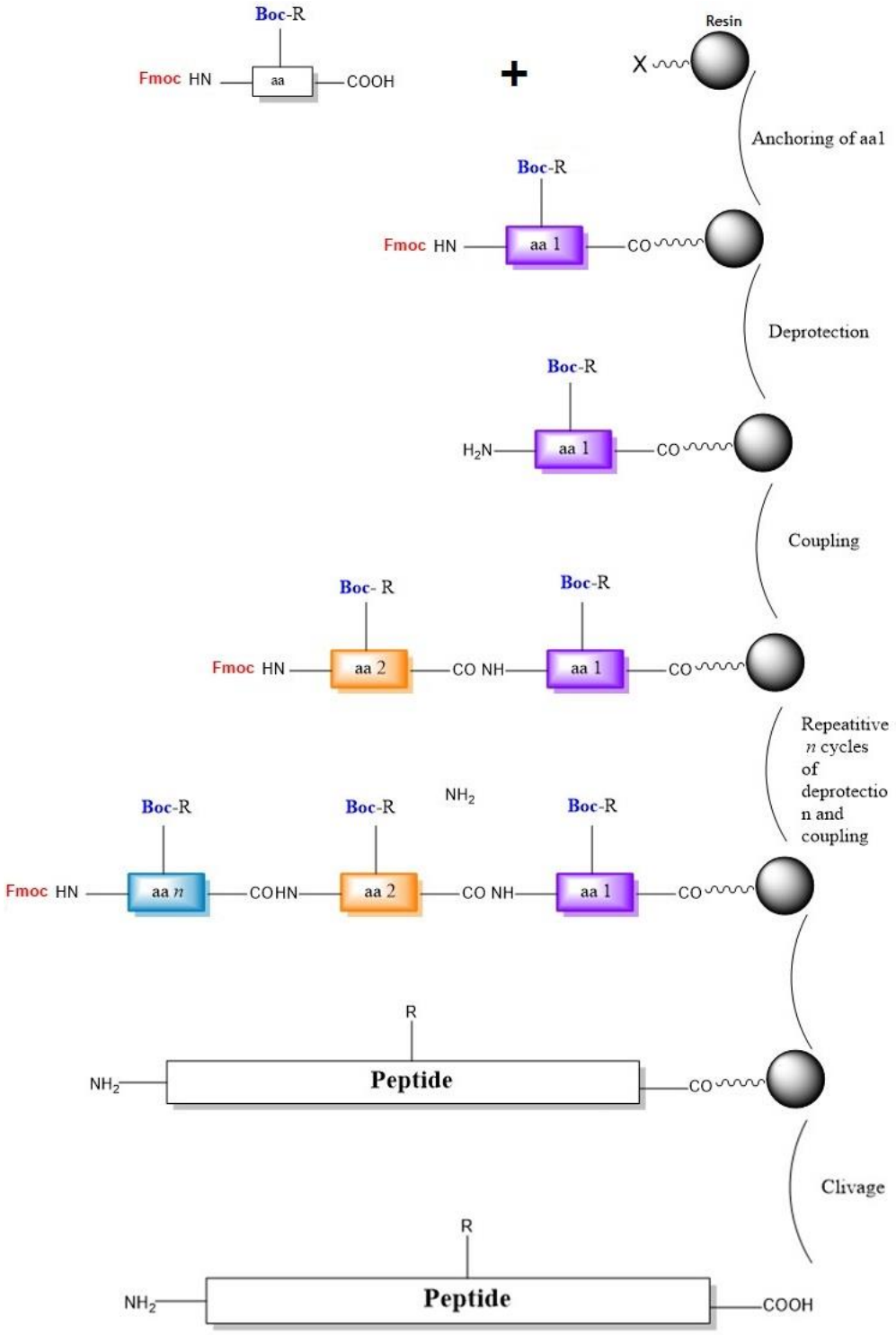


Figure 2.3- Scheme of main Steps of SPPS

Sample preparation

The peptide used in this work was t $C_{16}K_3$, composed by 3 lysines and palmitic acid as a tail, as shown in figure 2.4 and it was synthesised manually once the peptide is very small. It was synthesized on a 1 mmol scale, by SPPS

Lysine (abbreviated as **Lys** or **K**) is an amino acid with a very polar side chain that makes it highly hydrophilic. This amino acid is positively charged at neutral pH, since it contains amino groups (NH_2) which can be protonated (NH_3^+), so it is easier to penetrate into the cells as cell membrane is negatively charged.

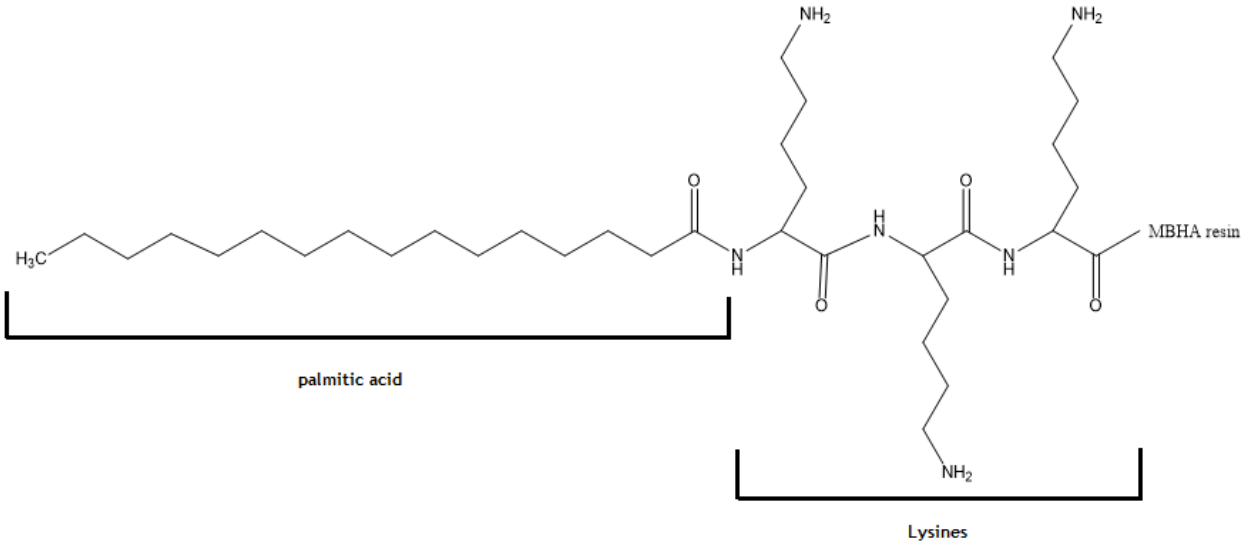


Figure 2.4 - Structure of C16K3 peptide attached to MBHA resin

This PA was already protected (Fmoc-Lys(Boc)-OH) and during its synthesis was washed with dimethylformamide (DMF) and dichlorometane (DCM) between each cycle of deprotection and coupling. At the end of each cycle, Kaiser test was performed. A blue color was obtained after each deprotection, meaning that they are free amines and yellow color at the end of each coupling ensuring that the amine group has been occupied.

The last step is called cleavage and was performed by acidolysis with strong acid, TFA (trifluoro acetic acid), using a cleavage cocktail composed by 2,5% of water, 2,5% of triisopropylsilane (TIS) and 95% of TFA. In this step, the resin was cleaved from the peptide and also the protecting groups of the side chain were removed, leaving the peptide free.

After that, is important to remove the remaining TFA of the previous step which can be toxic to cells. It was removed on a rotary evaporator under vacuum (850 mbar).

2.2. Peptide characterization and purification

After these steps, the free peptide was analysed by analytical high performance liquid chromatography (HPLC) and purified by preparative HPLC by reverse-phase method. For peptide identification, mass determination by electrospray ionization mass spectrometry (ESI-MS) was used.

2.2.1. High Performance Liquid Chromatography (HPLC)

HPLC is a technique able to separate the compounds in a mixture, identifying and quantifying each component.

This technique is based on a reservoir with solvents, a pump to move the solvent continuously creating a mobile phase with a desired flow rate through the column, an injector which inject the sample that is carried into the high pressure column (stationary phase) by the mobile phase stream, a detector, based on mass spectroscopy or UV for example, to observe the signals of each compound sending to a computer that records the signals and generate a chromatogram providing data analysis. [45]

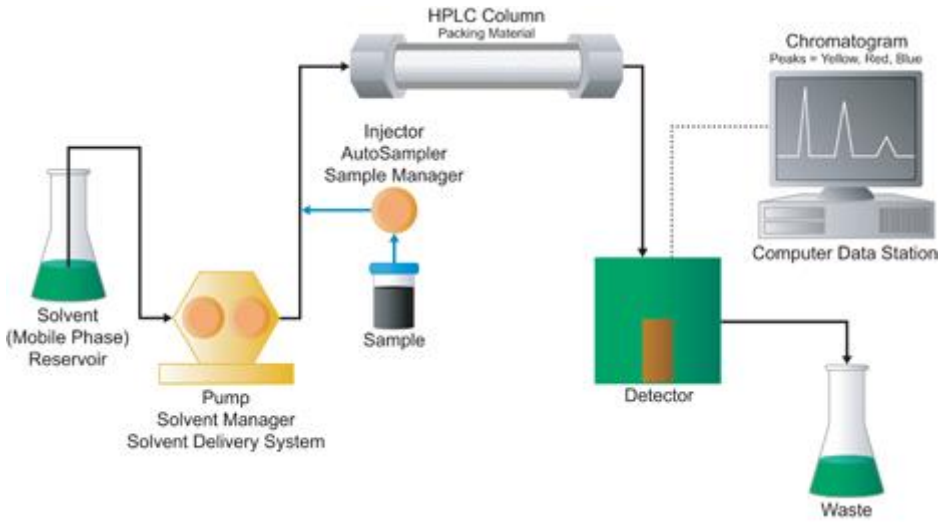


Figure 2.5 - Scheme of HPLC system. Adapted from [45]

The samples are liquid (solubilized in the solvent which is going to be used in mobile phase) and, before injected in the system, are filtered.

Once prepared, the sample is ready to run and when is injected it flows until reach the column leaving the mobile phase and enters in stationary phase where separation takes place depending on the mode used.

In the *Normal-Phase*, the stationary phase is polar (silica), relevant to substances insoluble in water. On the other hand, the *Reverse-Phase*, used in the present work, is the chromatography mode in which the stationary phase has low polarity and is used a polar mobile phase (water, buffer solutions, etc.) [45]

The *Reverse-Phase* has better resolution, higher viability to separate ionic samples, utilizes the column typically C18 which enables analysis from water-soluble and/or ionic substances to lipophilic substances.

The separation occur based on polarity of the compounds. Inside the column, first eluting polar substances which have lower interaction with apolar stationary phase.

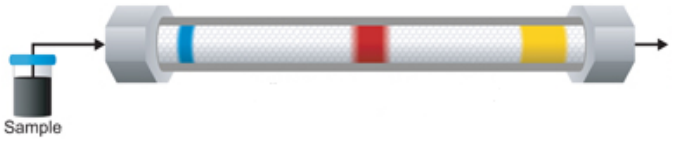


Figure 2.6 - Representation of a Reverse-Phase Chromatography column showing three analyte bands with different speeds of elution. Adapted from [45]

The figure 2.6 represent a sample with a mixture of yellow, red and blue dyes which moving at different speeds through the column, due to higher or lower attraction to the polar mobile phase. The yellow dye elute faster than the others due to it higher polarity and so, higher attraction to the solvent (mobile phase).

Besides of identification and quantification of compounds present in a mixture, this technique can also select desired amounts of each compound to purify the sample. This is performed in so-called preparative HPLC that operates in a higher scale than analytical HPLC. The preparative HPLC contains a fraction collector that collects purified fractions which are isolated from impurities.

Sample preparation

The $C_{16}K_3PA$, after being dissolved in water and freeze-dried, was prepared to be analysed in analytical HPLC A sample was dissolved in 0,1% TFA in water with a concentration of 1 mg/ml and filtered into specific vials.

A mass spectroscopic detector was used, which is based on chemical compounds that generate charged molecules by ionization which are separated on basis of their mass to charge. The column used was Waters XBridge column, designed to provide excellent conditions for acidic and basic mobile phases, and applied a flow rate of 1.0 ml/min.

The gradient used, Gene 45 minutes and the solvents injected for the first 32min was 0.1% TFA in water (98%) and 0.1% TFA in acetronitrile (2%), Which was Increasing until at Reached 100% at 35min. After this time, has been decreased to return to initial conditions.

In order to purify the peptide, the sample was run in preparative HPLC which was operated with a flow rate of 20.0 ml/min.

After that, the peptide undergone treatment processes with HCl to exchange TFA ions, coming from the preparative HPLC, followed by a dialysis to remove salts.

2.2.2. Electrospray Ionization Mass Spectrometry (ESI-MS)

For peptide identification and characterization, mass spectrometry is a powerful technique due to their sensitivity and since has the ability to provide highly accurate molecular weight information. [46]

The determination of mass is possible by ionization, without fragmentation, through different methods and occurs through the addition (positive ionization mode) or removal (negative ionization mode) of protons.

ESI-MS is composed by an ion source where the ions are produced and transferred in the mass analyser which are separated according to their mass to charge ratio (*m/z* value) and are detected in detector system to measure their concentration and a mass spectrum is displayed. [47] (figure 2.7 **a)**) The peaks on mass spectrum represents the intact molecule with variable charging, as shown in figure 2.7 **b)**.

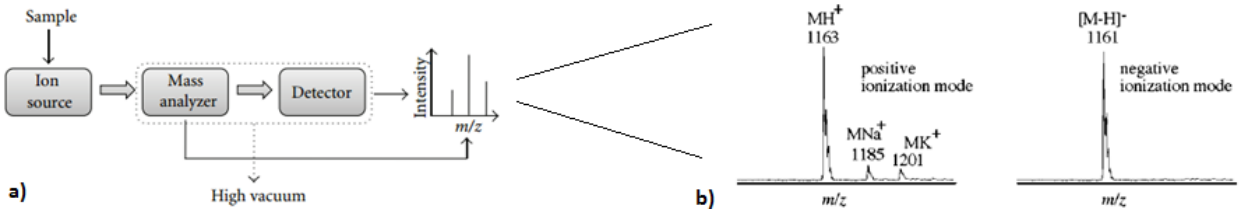


Figure 2.7 - a) Components of ESI-MS. All the components are kept at very high vacuum since the ions in gas-phase are very reactive and have short life [47]; **b)** Mass spectrum of a peptide in positive and negative ionization mode [46]

Sample preparation

The peptide, for mass analysis, was dissolved in methanol at to 0,1 mg/ml and filtered into specific vials and was performed before and after purification.

The masses of main possible fragments were previously calculated, to compare the values.

2.3. Critical micelle concentration

The purified peptide, when in aqueous solution, is able to form micelles since it is a surfactant, amphiphilic molecule, which contains a hydrophilic head and a hydrophobic tail.

At low concentration, the molecules line up on the air-water interface with the hydrophilic region in contact with water as shown in figure 2.8. Increasing the surfactant concentration, the interfacial tension, called surface tension, decrease and the surface becomes saturated leading to the micelle formation. [48]

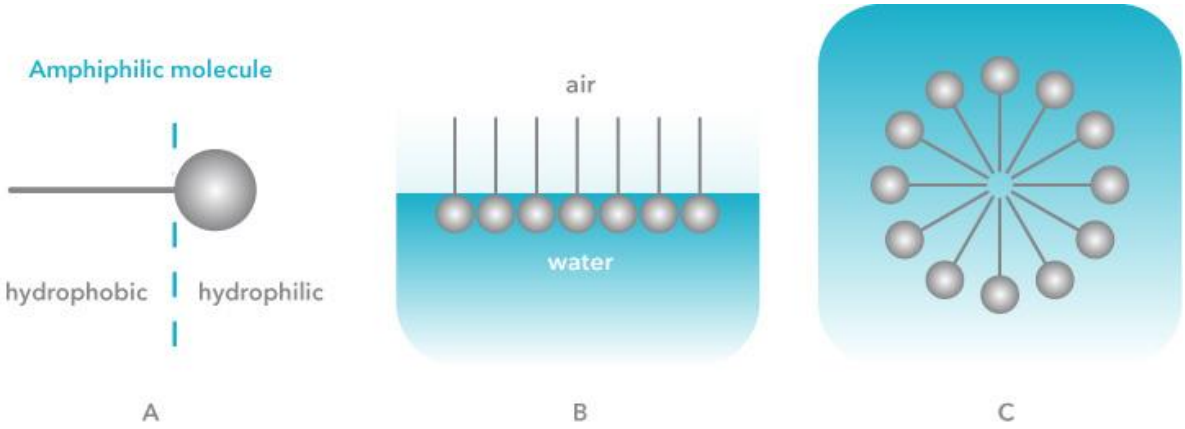


Figure 2.8 - Behaviour of amphiphilic molecule in aqueous environment. A) Structure of amphiphilic molecule; B) Amphiphilic molecule on air-water interface; C) Micelle with hydrophobic tail inside. Adapted from

This surfactant concentration above which micelles are formed is called critical micelle concentration (CMC) and it is important to study their stability. Many studies has been shown a reverse relationship between the CMC and hydrophobicity, concluding that the higher hydrophobic part length lower is CMC.[49]

According to the literature, generally, polymeric micelles show CMC values at around 10^{-6} or 10^{-7} M. [23]

There are different techniques to measure this concentration. In this work it was measured by fluorescence spectroscopy.

2.3.1. Fluorescence spectroscopy

Fluorescence is the emission of a photon, of lower energy, resulted when the molecule relaxes through vibrational relaxation and returns to ground electronic state, after being excited. So, when one molecule in the normal electronic state, the lowest vibrational level, absorb light is elevated to produce excited states. Then, the molecule can return to any level of the ground state, emitting its energy in the form of fluorescence. [50, 51] Figure 2.9 represents a diagram showing both absorption and fluorescence and levels of excited states.

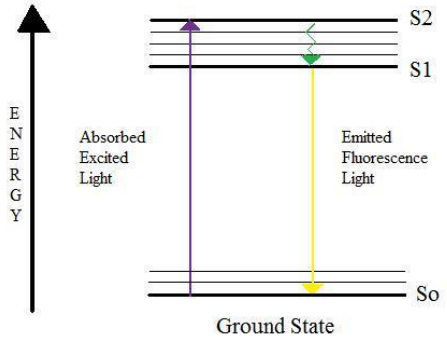


Figure 2.9 - Jablonski diagram representing the absorption of light (purple); vibrational relaxation, where no photon is emitted and the energy is dispersed to the solvent as heat or vibrations (green) and fluorescence (yellow)

This measurement was performed in a Fluorescence Spectrometer LS55, as shown in figure 2.10. A fluorimeter contains a source of light, two monochromator, a sample holder and a detector.

The radiation in the UV, visible or near-infrared regions is emitted in the lamp source, being the most commonly used aXenon arc lamp.

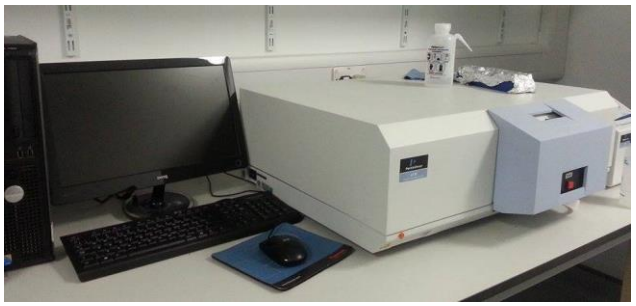


Figure 2.10 - Fluorescence Spectrometer LS55-QMUL

The light achieves first monochromator, which allows the preselection of the excitation wavelength, and passes through the sample holder where the sample is inside of a special cuvette. Then, the molecules in the solution after being excited emit light which is analysed by the emission monochromator.

The photomultiplier tubes, which are used as detector, receive the emitted light of the preselected wavelength and measure their intensity. [51]

The scheme, in figure 2.11, shows one example of instrument status.

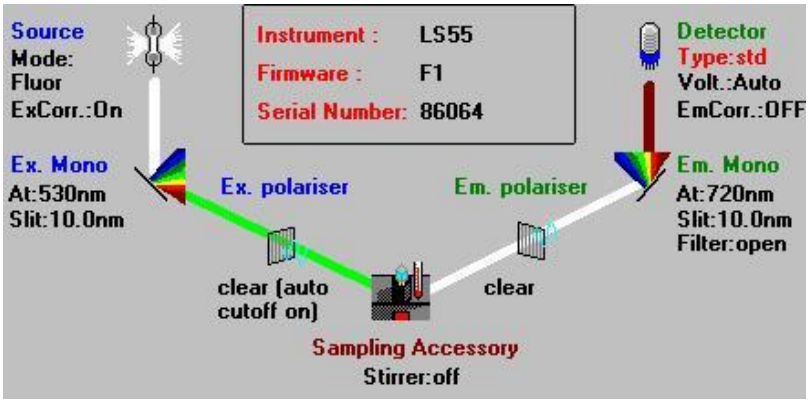


Figure 2.11 – Scheme of instrument status representing the individual components and current setup of the instrument

Sample preparation

The measurements on fluorescence spectrometer were performed by using Nile Red as a probe molecule in order to determine the CMC.

Nile Red has been recognized as a polarity-sensitive probe with high affinity to the hydrophobic regions. This fluorescent molecule, being poorly soluble in water, is expected to show weakly fluorescence in aqueous environment but a higher intensity at the CMC, due to micelles formation which permit a better solubilisation of the probe. [52, 53]

Nile Red was dissolved in methanol at 2 mM, diluted to 0,02mM and left overnight to evaporate the methanol. Several peptide samples were prepared at different concentrations, as shown in figure 2.12, dissolved in water and 100 µL of Nile Red solution was added in each sample to be analysed on fluorescence spectrometer.

All the steps were done in the dark due to the light sensitivity of the Nile Red.

All the samples were transferred into a cuvette to be measured on spectrometer.

The samples were excited at 530 nm and the range of emission wavelengths were included between 550 nm and 720 nm, since excitation/emission maxima ~552/636nm in methanol

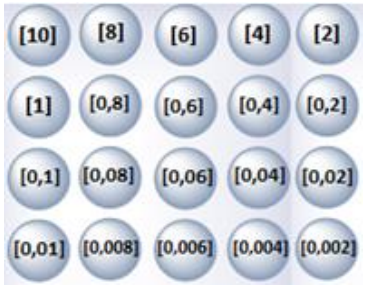


Figure 2.12 - Sample concentrations (mM) analysed by fluorescence spectroscopy

After the CMC is known, it is important to determine the size of the micelles and their stability, to obtain information on the performance of the micelles. These measurements were performed on Zetasizer Nano ZS equipment.

2.3.2. Zetasizer Nano ZS

Zetasizer is an instrument which provides the ability to measure three fundamental parameters of particles or molecules in a liquid medium.is used to measure particle and molecule size by dynamic light scattering technique, electrophoretic mobility of proteins, zeta potential of colloids and nanoparticles by electrophoretic light scattering technique and also enable the measurement of molecular weight by static light scattering technique.

Figure 2.13 - Malvern Instruments Zetasizer Nano ZS- QMUL

This work was performed in Zetasizer Nano ZS, as shown in figure 2.13.

2.3.2.1. Size Measurement by Dynamic Light Scattering

Determination of the size of nanoparticles in suspension is an important parameter to understand these materials in the nano-scale, being important maintaining their characteristics such as optical, chemical, thermal or electronic properties. [54, 55]

Particle size is considered to be the diameter of the sphere that diffuses at the same velocity of the particle which is measured. [56]

In this case, size is one of the most significant characteristic since only small particles can cross the BBB and measuring particle size distribution can be critical to reach the brain successfully.

This property is measured using a process called Dynamic Light Scattering (DLS) which uses a laser as a source of monochromatic light to illuminate the solution containing particles in Brownian motion. Then, the light, after passed through a collimator lens, which focus the beam, hits these particles causing a Doppler Shift and the light scatters in all directions. The scattered light passes through another collimator lens which ensures that the amount of light is the desired and is detected at a known scattering angle by a photomultiplier. Thus, the diffusion coefficient of the particles is measured with autocorrelation function and gives the information about the particles. [57, 58]

It is known that there is a relationship between speed of movement due to Brownian motion and the size of particles in a liquid (Stokes-Einstein equation). The particles in the sample are small if there is a quickly movement and the particle positions are quite different.

As the particles are constantly moving, the intensity of the scattered light appear fluctuate and Zetasizer system measures this fluctuation and uses it to calculate the size with a digital correlator. The correlation is related with time, comparing two intensity signals at different points in time, being 1 a perfect correlation, where the time is very short and the signals are identical and 0 when no correlation is observed. As mentioned above, the small particles move quickly and fluctuation will also faster than if the large particles are being measured. For that reason, the rate decay for the correlation function is related to particle size, as shown in figure 2.14.

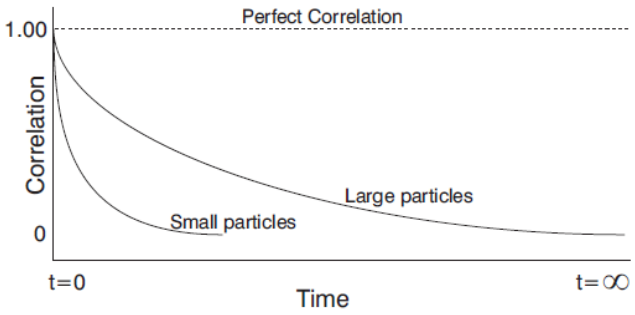


Figure 2.14 - Correlation function related with size of particles. Adapted from [56]

Then, with information of correlation function, the software uses algorithms to generate a size distribution, extracting the decay rates for a number of size classes. [56]

The results are presented in a size distribution by intensity graph, as shown in figure 2.15. It should show only one peak meaning that the sample contains only one size of particles.

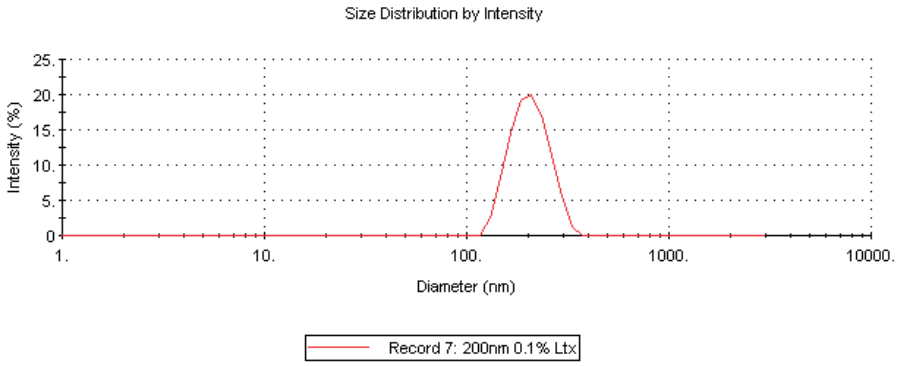


Figure 2.15 - Typical size distribution plot, showing size classes in X axis and the intensity of scattered light in Y axis. Adapted from [56]

Sample preparation

After determination of the CMC, a PA sample was prepared with a concentration higher than CMC value to ensure the micelles are formed.

The sample was dissolved in 1mL of water at 5 mM and was filtered into a clean cell to avoid any possible dust contamination, since this technique is very sensitive to the presence of aggregates or large contaminates which is not of interest or can cause result variations.

The cell was filled slowly to avoid air bubbles from being created.

2.3.2.2. Zeta Potential by Electrophoretic Light Scattering

The stability of the particles is another significant issue to study and this is done by measuring the zeta potential, determining the electrophoretic mobility, using laser Doppler velocimetry.

In a solution, the magnitude of electrostatic repulsion or charged between adjacent particles is called zeta potential, shown in figure 2.16,and gives an information about the potential stability of the colloidal system.

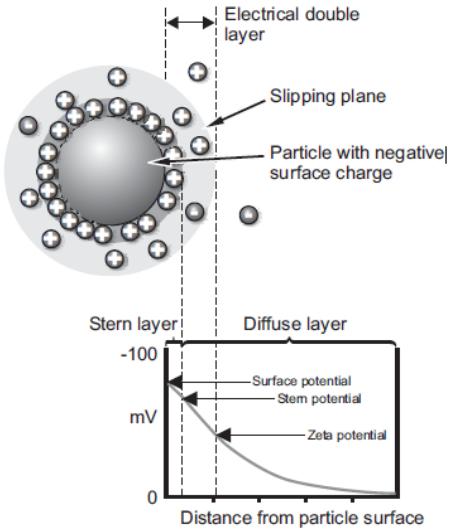


Figure 2.16 - Scheme of the charged surface of a particle in aqueous medium. Adapted from [59]

Thus, when the zeta potential value is low, attractive forces may exceed the repulsion and the dispersion tends to coagulate or flocculate. Otherwise, particles with high values of zeta potential, negative or positive, are considered stable, mainly > +30 mV or <-30 mV, as shown in table below. [60-62]

Table II - Zeta potential values and correspondent stability behavior of the colloid. Adapted from [61]

Zeta Potential (mV)	Stability
0 to ± 5	Rapid coagulation or flocculation
±10 to ±30	Incipient instability
±30 to ±40	Moderate Stability
±40 to ±60	Good Stability
> ±61	Excellent Stability

Electrophoretic Light Scattering (ELS) is based on DLS but, here, the frequency shift is caused by oscillating electric field. [63]

When an electrical field is applied, the charged particles move towards the oppositely charged electrode and their velocity is measured and expressed in unit field strength as their mobility. [62, 64]

This is called electrophoresis and this velocity is measured by analysing resultant light intensity frequency by the seeded particles within the flow, as shown in figure 2.17.

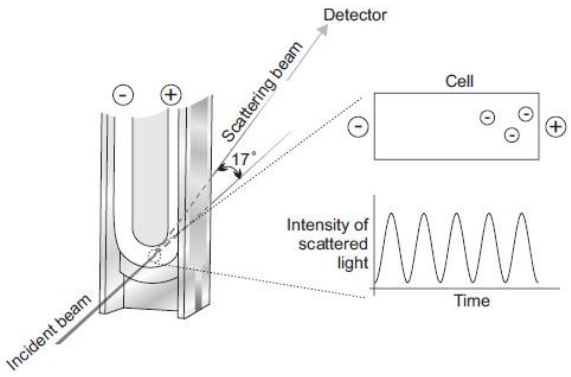


Figure 2.17 - Measurement of velocity of particles moving through a fluid in an eletrophoresis experiment by laser doppler velocimetry

Once known the velocity of the particle and the electrical field applied, it is possible measure the zeta potential using the viscosity and dielectric constant (Henry equation):

$$U_E = \frac{2\,\varepsilon\,\zeta f(ka)}{3\eta}$$

- ζ: zeta potential
- U_E: Eletrophoretic mobility
- ε: Dielectric constant
- η: Viscosity
- f(Ka): Henrys function

Sample preparation

The sample, in this case, was also filtered, eliminating sample cross contaminations, but transferred into a special cell with two electrodes, disposable folded capillary cell (DTS1060), shown in figure 2.18, to measure the electrophoretic mobility of the particles in solution.

The sample, at same concentration used in size measurements (5 mM), was transferred to the cuvette carefully, using syringes, to avoid bubble formation inside the cell and measurement errors.

The sample was slowly injected through one of electrodes and covering the other electrode with one cap, when the sample starts to emerge. This cap ensures greater thermal stability of the sample and can prevent dust contamination and possible spillage. After checking that there were no bubbles, the other electrode was also covered with other cap.

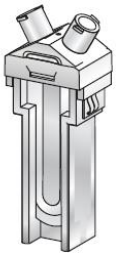


Figure 2.18 – Disposable folded capillary cell.(DTS1060) Adapted from [56]

Chapter 3

Results and discussion

3.1. Peptide Synthesis and characterization

$C_{16}K_3$ PA was successfully synthesized, following Solid-Phase Peptide Synthesis protocol, purified using preparative HPLC and confirmed by analytical HPLC.

After the first deprotection to remove Fmoc group, the Kaiser test was shown blue dark, meaning the amine group was free, ready to couple the next amino acid. The yellow colour was observed after the test, carried out after the first coupling to ensure that was well-done. The yellow colour means that the amine group was occupied and the coupling was done as desired. The same happened for the following amino acids, except for the tail which was more difficult to attach to the peptide.

Figure 3.1 shows the analytical HPLC chromatogram before purification and figure 3.2 after purification.

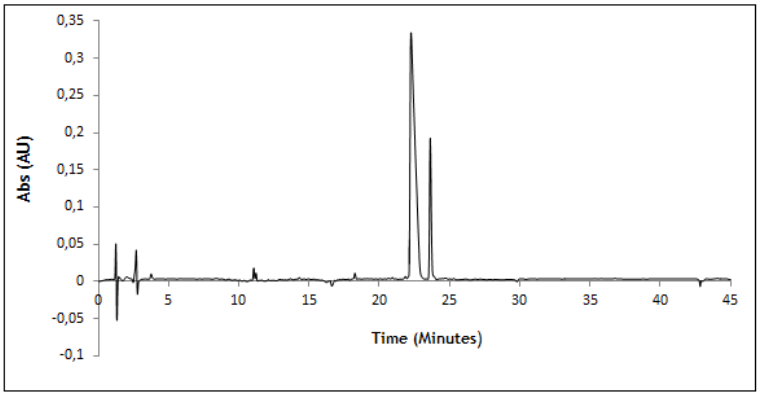


Figure 3.1 – Chromatogram (analytical HPLC) of the crude peptide

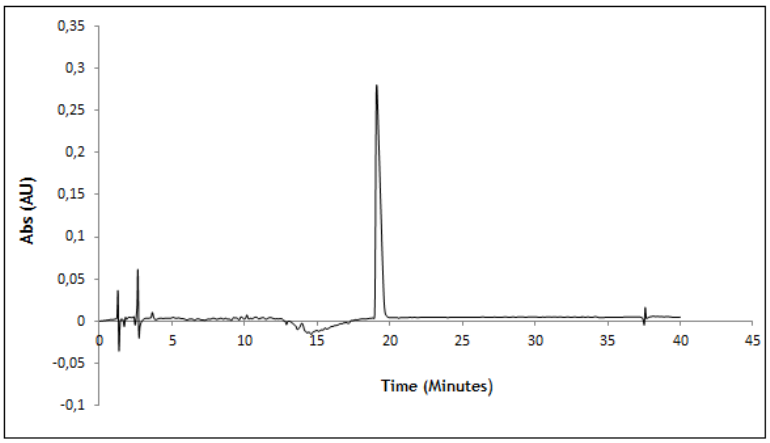


Figure 3.2 – Chromatogram (analytical HPLC) of the pure peptide

In the analytical HPLC, after purification, it was observed only one peak, representing the peptide in the sample, concluded that the peptide was with high purity, since no other components were observed in the sample.

The retention time was around 20 min which means that the peptide takes this time to elute from the column after injection. According to the gradient used, C16K3 PA only started to leave the column with the addition of 0,1% TFA+ acetronitrile through the column. This means that the peptide, despite being water soluble, didn't elute early, when the solvent was totally polar (0,1%TFA in water), due to the hydrophobicity of the peptide tail which has affinity to apolar stationary phase.

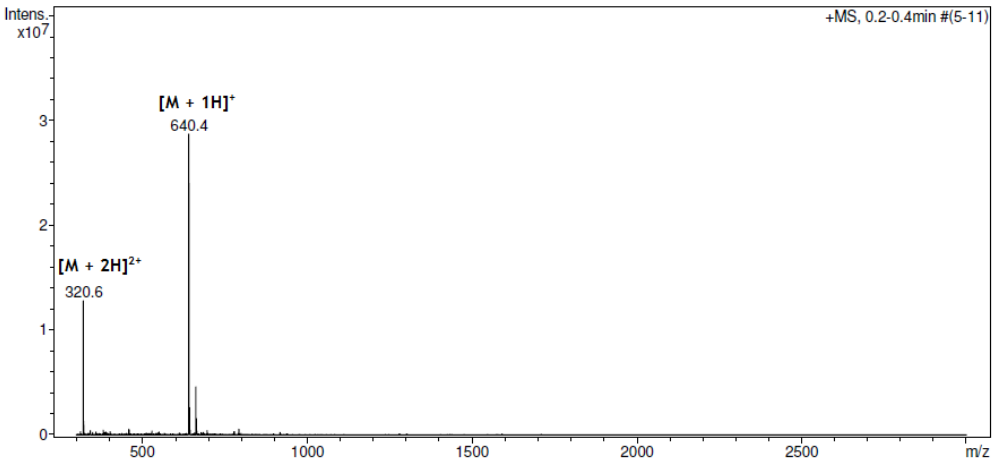


Figure 3.3 – Mass spectrum of $C_{16}K_3$ PA, after peptide purification

Table III – Expected and observed mass to charge ratio of the main fragment ions of $C_{16}K_3$ peptide

Peptide	**Molecular weight (g/mol)**			
$C_{16}K_3$	639.97	$[M+1H]^+$	$[M+2H]^{2+}$	$[M+3H]^{3+}$
Expected mass (m/z)		640.97	320.99	214.32
Observed mass (m/z)		640.4	320.6	____

The results of mass spectrometry is a spectrum, where are represented peaks characterized by mass-to-charge ratio (m/z) of smaller peptide fragments, coming from ionization, and intensity value which is the number of detected fragments.

In figure 3.3, it is possible to see, two main peaks meaning that the peptide have one and two sites of protonation. The first peak, with 320.6 m/z, represents a doubly charged fragment ion, i.e., the charge state of the fragment is +2 and the second peak, with 640.4 m/z means that less fragments are

singly charged (+1). These values were the expected for this peptide, previously calculated, as shown in table III.

3.2. Fluorescence Spectroscopy

The CMC was evaluated by fluorescence spectroscopy and due to the lower water solubility of Nile red, shifts in the fluorescence intensity and corresponding emission maximum wavelength were observed. For C=CMC, there is a sharp increase in fluorescence intensity and a hypsochromic effect (blue shift). This blue shift, a decrease in wavelength with a corresponding increase in frequency was observed in figure 3.4.

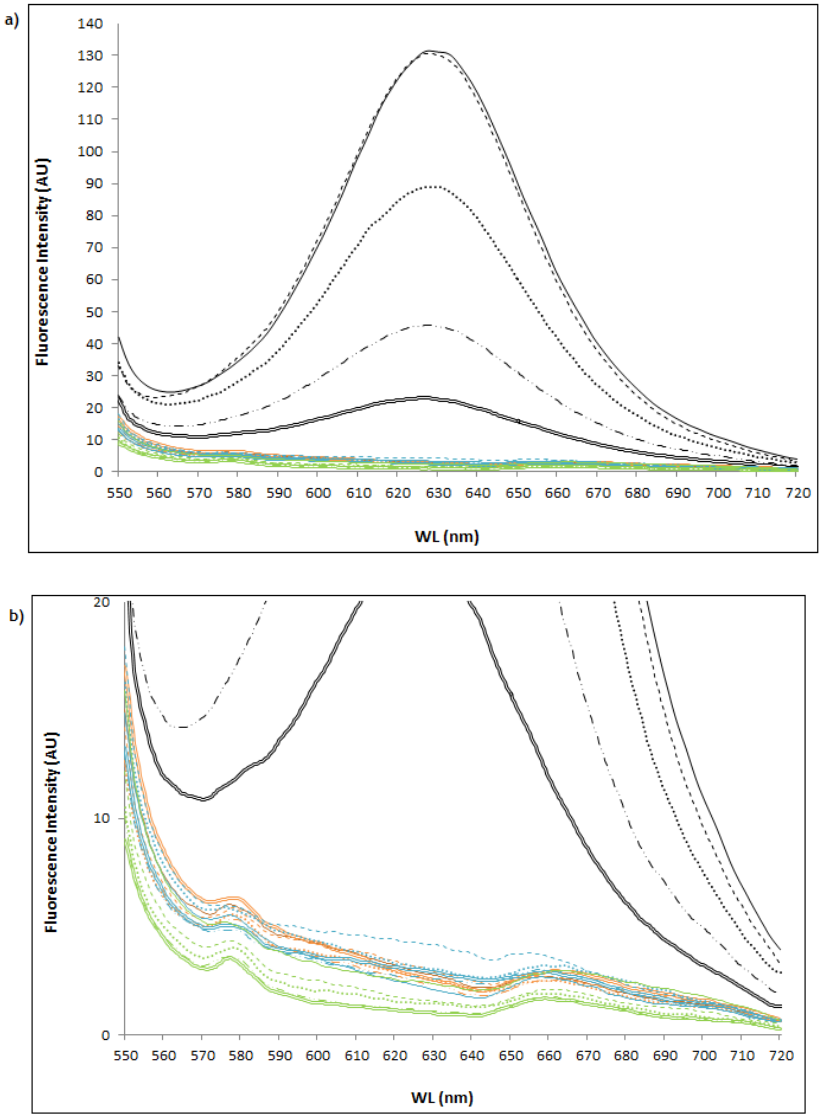


Figure 3.4 - Fluorescence measurements of samples with concentrations between 0,002 mM and 10 mM showing different fluorescence intensity scale, **a)** in a range 0-140 AU; **b)** 0-20 AU

Observing the graphs of fluorescence, it is clear a change in intensity in fluorescence for samples with higher concentrations. This occurs because at these concentrations, the PA starts to form micelles in aqueous environment and, due to its hydrophobicity, the used dye is incorporated into micelle pocket.

To measure the CMC point, the concentration of the different samples was plotted against the maximum intensity and correspondent wavelength (figure 3.5), suggesting a CMC value around 4 mM, meaning micelle formation only from this value. This CMC value is lower than the other systems previously studied, such as polymeric micelles, in the range between 10^{-6} or 10^{-7} M of, mentioned in the previous chapter.

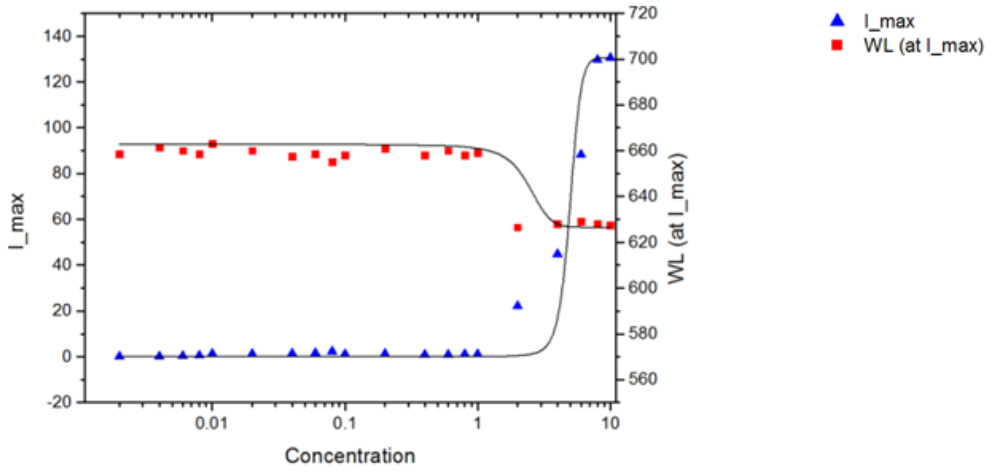


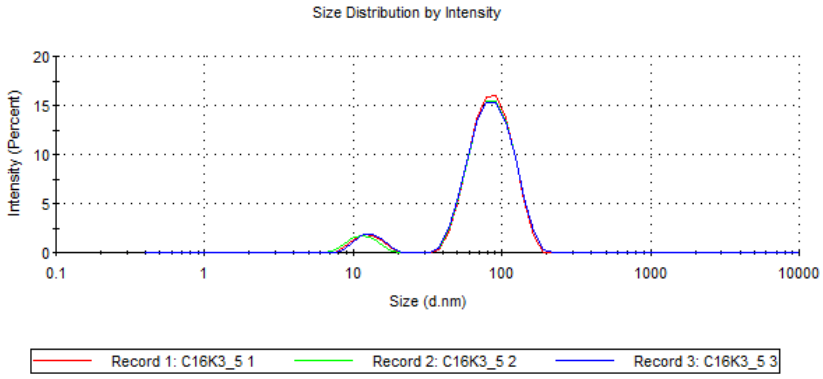
Figure 3.5 - Offset of I_max and WL at I_max vs Log(Concentration at mM), using OriginPro 9 software

3.3. Zetasizer nano ZS

3.3.1. Size measurements

The results of size measurement are shown in figure 3.6 with two peaks meaning that there were particles with two different sizes in the sample.

Since the length of the $C_{16}K_3$ PA molecule is around 5 nm, estimated in ChemDraw software, and based on other studies with peptide amphiphile micelles, is possible say that the small peak at around 12 nm is about micelle structure and the larger one can be vesicles forming also by self-assembly.



	Size (d.nm):	% Intensity:	St Dev (d.nm):
Peak 1:	88.87	93.1	28.49
Peak 2:	12.99	6.9	2.471
Peak 3:	0.000	0.0	0.000

Figure 3.6 – Intensity stands for particle size distribution and the details of the peaks

This result shows controlled micelle size and small enough for drug delivery into the brain, since less than 100 nm is ideal to cross the BBB.

3.3.2. Zeta potential measurements

The value of zeta potential measurement was 60.8 mV, meaning, according table II of the previous chapter, a good stability of the micelles.

Chapter 4

Conclusions and future works

The $C_{16}K_3$ PA, successfully synthesized and purified, was able to form micelles, easily, in aqueous environment, showing low CMC values, which is one advantage of these systems, as well as, their great stability in solution and appropriate size for drug delivery into the brain.

The fact of the peptides amphiphile are easy to synthesize, forming micelles easily, by self-assembly, stable and small enough to cross the BBB, and well organized structure, with multiple domains, makes them a good candidate to act as nanocarrier to transport drugs and reach the brain.

To assist their transport and minimizing their side effects, these systems are advantageous over the others already developed, previously mentioned in this thesis, due to the possible incorporation of targeting sequences into the peptide, without further complex modifications on surface.

For future works, the next step will be the loading of the drug into $C_{16}K_3$ PA and the study of drug release in order to better understand the mechanism and to ensure a controlled release.

In order to assist their transport across the BBB and reach the target sites, will be incorporated targeting sequences, for instance, ligand for specific receptors of BBB which can be overexpressed in pathological conditions.

And, finally, testing the peptide as nanocarrier, in vitro, mimicking biological conditions with BBB models.

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